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A Novel Pineal-specific Product of the Oligopeptide Transporter *PepT1* Gene

CIRCADIAN EXPRESSION MEDIATED BY cAMP ACTIVATION OF AN INTRONIC PROMOTER*[S]

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The oligopeptide transporter 1, PepT1, is a member of the Slc15 family of 12 membrane-spanning domain transporters; PepT1 has proton/peptide cotransport activity and is selectively expressed in intestinal epithelial cells, where it is responsible for the nutritional absorption of di- and tri-peptides. Here, a novel *PepT1* gene product has been identified in the rat pineal gland, termed pgPepT1. It encodes a 150-amino acid protein encompassing the C-terminal 3 membrane-spanning domains of intestinal PepT1 protein, with 3 additional N-terminal residues. Expression of pgPepT1 appears to be restricted to the pineal gland and follows a marked circadian pattern with >100-fold higher levels of mRNA occurring at night; this is accompanied by an accumulation of membrane-associated pgPepT1 protein (~16 kDa). The daily rhythm in pgPepT1 mRNA is regulated by the well described neural pathway that controls pineal melatonin production. This includes the retina, the circadian clock in the suprachiasmatic nucleus, central structures, and projections from the superior cervical ganglia; activation of this pathway results in the release of norepinephrine. Here it was found that pgPepT1 expression is mediated by a norepinephrine → cyclic AMP mechanism that activates an alternative promoter located in intron 20 of the gene. pgPepT1 protein was found to have transporter-modulator activity; it could contribute to circadian changes in pineal function through this mechanism.

A defining characteristic of the vertebrate pineal gland is a global 24-h pattern of activity, as best exemplified by the nocturnal increase in melatonin production. The resulting increase in circulating melatonin provides a signal of night time and is used to optimally synchronize physiological functions with daily changes in environmental lighting (1). In addition to this role in daily rhythms, the melatonin signal is essential for the integration of seasonal photoperiodic changes in physiology, thereby influencing the timing of reproduction and associated physiological and behavioral changes.

The 24-h pattern of most aspects of pineal function is driven by an endogenous ~24-h oscillator (2); in mammals, this oscillator is located in the anterior hypothalamus above the optic chiasm, in the suprachiasmatic nucleus (SCN[1]) (3). Light synchronizes this oscillator with the environmental light schedule via a neural projection from the retina to the SCN. The SCN clock is linked to the pineal gland by a multisynaptic pathway, involving the hypothalamic paraventricular nucleus, the intermediolateral cell column of the spinal cord, and the peripheral sympathetic nervous system (4); light also acts to terminate SCN stimulation of the pineal gland. The sympathetic cells, innervating the pineal gland, are located in the superior cervical ganglion (SCG). Activation of the SCN-pineal pathway at night results in the release of norepinephrine (NE) into the pineal perivascular space. Circadian release of NE occurs also in continuous darkness, reflecting the autonomous nature of the SCN clock.

Rhythmic melatonin production involves cyclic AMP-dependent stimulation of the activity of the penultimate enzyme in the melatonin synthesis pathway, arylalkylamine-*N*-acetyltransferase (AANAT); large changes in the activity of this enzyme drive the large rhythm in melatonin synthesis (5). In rodents, the regulation of AANAT occurs at the transcriptional level, with a >100-fold diurnal change in mRNA levels (6), and at the post-translational level, where it involves phosphorylation events (7).

During the past two decades, the list of genes that are rhyth-

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[S] The on-line version of this article (available at http//www.jbc.org) contains Supplemental Fig. S1.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY860424.

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[1] The abbreviations used are: SCN, suprachiasmatic nucleus; pgPepT1, pineal gland PepT1; AANAT, arylalkylamine *N*-acetyltransferase; HIOMT, hydroxyindole-*O*-methyltransferase; CCAATinv, inverted CCAAT box; Crx, cone rod homeobox protein; CRE, cAMP-responsive element; LD, light:dark; DD, constant darkness; LL, constant light; NE, L-(−)-norepinephrine; ISO, (−)-isoproterenol; Bt_2cAMP, dibutyryl cyclic AMP; PCE, photoreceptor conserved element; SCG, superior cervical ganglion; SCGX, bilateral cervical ganglionectomy; DCNT, decentralization; TMD, transmembrane domain; Gly-Sar, glycylsarcosine; ZT, Zeitgeber time; 5′-RACE, rapid amplification of 5′ cDNA ends; DMEM, Dulbecco's modified Eagle's medium; nt, nucleotide(s); CHO, Chinese hamster ovary.

mically expressed in the rat pineal gland has grown; it includes genes encoding transcription factors (8, 9), enzymes (10), transporters (11), and receptors (12–14). This list continues to grow, as a result of the application of DNA microarray methods (15–20). As a result, it is becoming clear that there is a global increase in gene expression at night in the rat and chicken pineal gland.

A recent microarray analysis has revealed that there is a ~60-fold night/day difference in the abundance of PepT1 mRNA in the rat pineal gland.[2] The *PepT1* gene encodes a 12 membrane-spanning domain protein, PepT1 (member 1 of the solute carrier family 15, Slc15a1), which has proton/peptide cotransporter activity (21). This protein is strongly and relatively selectively expressed in the small intestine and has a physiological role in nutrient transport through the efficient absorption of di- and tri-peptides arising from breakdown of food protein; it also has a pharmacological significance, mediating the transport of peptide-like drugs (21). Here, we describe results of studies that reveal that the highly rhythmic pineal *PepT1* gene product (pgPepT1) is novel and that expression is neurally controlled by an alternative intronic promoter. The results of these studies point to a potential role of the encoded protein in daily modulation of pineal metabolism.

EXPERIMENTAL PROCEDURES

Materials—L-(−)-Norepinephrine (NE), (−)-isoproterenol (ISO), *R*-(−)-phenylephrine, dibutyryl cyclic AMP (Bt_2cAMP), KT5720, and (R_p)-8-PIP-cAMP were purchased from Sigma. Forskolin and H-89 were purchased from Calbiochem.

Animals and in Vivo Experiments

Rats used for *in situ* hybridization studies (Figs. 2*B*, 2*C*, and 4*B*) were Wistar rats obtained from the Panum Institute (Copenhagen, Denmark) and were housed for 2 weeks in a controlled lighting environment (12 h of light, 12 h of darkness; LD 12:12). Otherwise, Sprague-Dawley rats were used; surgically prepared animals were from Zivic-Miller laboratories (Allison Park, PA), and other animals were from Taconic Farms Inc. (Germantown, NY). These animals were housed in an automatically regulated lighting cycle (LD 14:10) except in experiments in which animals were kept under constant darkness (DD) or constant light (LL) for 3 days. For *in vivo* drug treatments, rats were injected subcutaneously at the nape of the neck with 1 mg/kg NE (Fig. 4*A*) or intraperitoneally with 5 mg of ISO (Fig. 4*B*). Rats were killed by CO_2 asphyxiation and decapitation at the indicated Zeitgeber time (ZT). During the dark period, animals were killed under red safe lights. Tissues were removed, immediately placed on dry ice, and stored at −80 °C until use. All procedures were done according to NIH guidelines.

DNA Cloning

Cloning of Rat pgPepT1 cDNA—Clones of pgPepT1 were obtained by the rapid amplification of 5′ cDNA ends (5′-RACE) strategy from rat pineal cDNA. Briefly, solid phase cDNA synthesis on magnetic oligo(dT) beads (Dynal, Lake Success, NY) was done using 10 μg of total RNA from rat pineal glands collected at midnight (22). After dC-tailing of the cDNA, PCR was done with an antisense primer specific of the rat PepT1 cDNA sequence (GenBank™ accession number NM057121, nucleotides (nt) 2262–2281) and an anchor-poly-dG primer. The PCR product was subcloned into pGEM-T easy vector (Promega Corp., Madison, WI) and sequenced. For pgPepT1 protein expression studies, the coding region of pgPepT1 cDNA was cloned into pcDNA3.1(+) expression vector (Invitrogen), which places the cDNA under the control of the cytomegalovirus promoter.

Promoter-luciferase Reporter Plasmid Construction—A fragment of the putative intronic pgPepT1 promoter (intron 20 of *PepT1* gene; GenBank™ accession number NW047456) was generated by PCR amplification using high fidelity *Pfu* DNA polymerase (Stratagene, La Jolla, CA), with rat genomic DNA as a template. A sense primer (position relative to the pgPepT1 transcription start site: nt −417 to −397), including a NheI restriction site, and one antisense primer (nt +27 to +45), including a XhoI restriction site were used to amplify a 462-bp promoter fragment (intron20, −417/+45) (Fig. 6*B*). Another sense primer (nt −289 to −269) was also used to amplify a 334-bp promoter fragment (intron20-PCE, −289/+45), which does not contain the three photoreceptor conserved elements (PCE) (Fig. 6*B*). After digestion by NheI and XhoI, the promoter fragments were subcloned into the promoterless pGL3-basic vector (Promega), upstream of the firefly luciferase coding sequence (Fig. 6*B*).

Culture Methods

Pineal Organ Culture—Adult female Sprague-Dawley rats were decapitated, and the pineal glands were collected and immediately placed in organ culture (6, 23).

Cell Culture—Pinealocytes were prepared from rat pineal glands, which were collected and rinsed in 10 ml of Earle's balanced salt solution (Worthington). The dissociation medium, containing 20 units/ml papain and 200 units/ml DNase I (Worthington) in Earle's balanced salt solution, was pre-activated for 30 min at 37 °C. Glands were incubated in this dissociation medium for 50 min at 37 °C (95% air to 5% CO_2), then triturated twice and incubated for an additional 10 min. Digestion was ended by addition of 1 ml of fetal calf serum. The preparation was spun at 1000 × *g* for 3 min, and the cell pellet was resuspended and triturated in 5 ml of Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. The pineal cell preparation was passed through a 70-μm cell strainer (BD Falcon, Bedford, MA) and spun at 1000 × *g* for 10 min. The cell pellet was then resuspended in DMEM media supplemented with 5 or 10% fetal calf serum. This procedure is a modification of a published method (24). CHO-K1 cells (ATCC, Manassas, VA) were cultured as a monolayer at 37 °C (95% air to 5%CO_2) in Ham's F-12K medium (ATCC) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. COS-7 cells (ATCC) were cultured as a monolayer in DMEM supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C (95% air to 5% CO_2).

DNA Transfection—CHO-K1 and COS-7 cells were plated 1 day before transfection onto 24-well plates at a density of 5×10^4 cells/well. Transfections were performed using Lipofectamine (Invitrogen), according to the manufacturer's instructions. Cells were transfected overnight in Opti-MEM I medium (Invitrogen). The next day, the media was replaced by complete F-12K or DMEM media and treated as indicated. Twenty-four hours following initiation of treatment, the cells were collected.

Pinealocytes were plated at a density of 5×10^5 cells/well in 24-well plates (Corning Inc., Acton, MA), in 400 μl of DMEM supplemented with 5% fetal calf serum. Cells were transfected by adding 100 μl of Opti-MEM I medium containing 2 μl of Lipofectamine 2000 (Invitrogen) and 0.8 μg of DNA to each well. The cells were stimulated as described in the text and collected 48 h post-transfection.

Analytical Techniques

Northern Blot Analysis—PepT1 probes, covering either the 5′ region (nt 369–1525) or the 3′ region (nt 1838–2281) of the rat PepT1 mRNA (GenBank™ accession number NM057121) were obtained by PCR amplification on pSPORT plasmid containing the full-length rat PepT1 cDNA (provided by Dr. Ken-Ichi Inui, Kyoto University Hospital (25)) using the primer pair (sense: nt 369–388; antisense: nt 1506–1525) or the primer pair (sense: nt 1838–1857; antisense: nt 2262–2281), respectively. All hybridization probes were ^{32}P-labeled by random priming using Ready-To-Go DNA labeling beads (−dCTP) kit (Amersham Biosciences). Total RNA were isolated using TRIzol reagent as described by the manufacturer (Invitrogen), resolved on a 1.5% agarose/0.7 M formaldehyde gel, transferred to a charged nylon membrane by passive capillarity transfer, and immobilized by UV cross-linking. Blots were hybridized at 68 °C for 2 h in QuikHyb buffer (Stratagene). The final wash was in 0.1× standard saline citrate (SSC) containing 0.1% SDS at 60 °C for 20 min. Blots were imaged and quantitated using a PhosphorImager (Amersham Biosciences). Values were normalized to the signals generated by ribosomal 18 or 28 S RNA. Transcript sizes were estimated by comparison with RNA markers (Roche Applied Science).

In Situ hybridization—Sagittal and coronal cryostat sections of rat brains were cut (15-μm thick) and thaw mounted on Superfrost Plus® glass slides. The slides were kept at −80 °C until processed. The sections were hybridized as previously described (13), with an ^{35}S-labeled 38-mer oligonucleotide probe derived from the rat PepT1 mRNA se-

[2] *PepT1* gene expression in the rat pineal gland was 62-fold higher at midnight relative to midday as detected using Affymetrix RG34A oligonucleotide microarray Genechip (Humphries, A., Carter, D., Coon, S. L., Munson, P. J., and Klein, D. C., unpublished).

quence (GenBank™ accession number NM057121; nt 1996–2033, antisense). Briefly, the probe (25 pmol diluted in sterile diethyl pyrocarbonate-treated and water) was labeled with [^{35}S]ATP and terminal transferase (Roche Applied Science) to a specific activity of 1×10^{18} dpm/mol. Frozen tissue sections were then thawed and fixed for 5 min in 4% paraformaldehyde in phosphate-buffered saline and washed 2 × 1 min in phosphate-buffered saline. This was followed by acetylation of the sections in 0.25% acetic anhydride (diluted in 0.1 M triethanolamine and 0.9% NaCl) for 10 min. The sections were then dehydrated in a graded series of ethanol and delipidated in 100% chloroform (5 min). They were partially rehydrated in 100 and 95% ethanol (1 min each) and allowed to dry. For hybridization, labeled probe was diluted in the hybridization buffer (10 μl of labeled probe/ml of hybridization buffer) consisting of 50% (v/v) formamide, 4× SSC (150 mM NaCl, 15 mM sodium citrate, pH 7.0), 1× Denhardt's solution (0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 0.02% Ficoll), 10% (w/v) dextran sulfate, 10 mM dithiothreitol, 0.5 mg/ml salmon sperm DNA, and 0.5 mg/ml yeast tRNA. An aliquot of 200 μl of the labeled probe was pipetted onto each section. The sections were then covered with Parafilm® and incubated in a humid chamber overnight at 42 °C. After hybridization, the slides were washed in 1× SSC for 4 × 15 min at 55 °C, 2 × 30 min at room temperature, and rinsed twice in distilled water. The sections were dried and either exposed to an x-ray film for 1–2 weeks or dipped into Amersham Biosciences LM-1® emulsion and exposed for 2–4 weeks at 4 °C.

Tissue Homogenate Preparation and Western Blot Analysis

Crude membrane fractions were prepared as previously described (26). Briefly, each tissue was homogenized in a buffer composed of 0.23 M sucrose, 5 mM Tris-HCl, pH 7.5, 2 mM EDTA, and protease inhibitor mixture (Complete, Roche Applied Science). The homogenate was first centrifuged at 3,000 × *g* for 15 min, and the supernatant was further centrifuged at 100,000 × *g* for 30 min. The resultant pellet, referred to as the crude membrane fraction, was solubilized in loading buffer and subjected to standard polyacrylamide gel electrophoresis on a 14% precast SDS-PAGE gel (Novex, Invitrogen). Proteins were transferred onto Immobilon membranes (Millipore Corp., Bedford, MA) by semidry electroblotting. The blots were immunodetected as previously described (27), using anti-rat PepT1 serum (1:1000) raised against the 15 C-terminal amino acids of rat PepT1 (provided by Dr. Ken-Ichi Inui, Kyoto University Hospital (25)). Uniform loading was confirmed using anti-synaptophysin serum (monoclonal clone SVP-38, Sigma). The blots were exposed to BioMax Light or MR film (Eastman Kodak, Rochester, NY) and quantitated using ImageQuant 5.2 software (Amersham Biosciences); values were normalized using the immunosignals obtained with the anti-synaptophysin serum.

Luciferase Assay

Transfected cells were assayed for luciferase activity, using the Dual-Luciferase reporter assay system (Promega), according to the manufacturer's instructions.

Uptake Analysis

The ability of pgPepT1 to transport glycylsarcosine (Gly-Sar), a non-hydrolyzable substrate for the peptide transporters PepT1 and PepT2, was investigated using a mammalian cell expression system in human retinal pigment epithelial cells (28). The vaccinia virus expression technique was used for this purpose. hPepT1 and mPepT2 were expressed with or without pgPepT1 to determine their potential interaction with pgPepT1. The cloning and functional characterization of human PepT1 has been described previously (28). Mouse PepT2 was cloned from a kidney cDNA library. pgPepT1 cDNA was present in pcDNA3.1, whereas hPepT1 and mPepT2 cDNAs were present in pSPORT1. Therefore, respective empty plasmids were used to maintain the amount of plasmid DNA used in each assay (2 μg/assay). Transport activity was measured in these cells 15 h following transfection. Transport of [^{14}C]Gly-Sar (50 μM) was measured for 15 min at 37 °C in a buffer (pH 6) containing 25 mM 4-morpholineethanesulfonic acid/Tris, 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 5 mM glucose.

RESULTS

pgPepT1 Is a Novel PepT1 Gene Product Expressed at Night in the Pineal Gland—The nocturnal induction of *PepT1* gene expression in the pineal gland, first revealed by microarray studies was confirmed here by Northern blot analysis using a probe specific for the region between nt 1838 and 2281 of the rat intestinal PepT1 cDNA (Fig. 1*A*). The pineal transcript (pgPepT1, 1.5 kb) is approximately one-half the size of that in the intestine (3 kb (25)). pgPepT1 was not detected by a probe directed against the 5′ region (nt 369–1525) of the PepT1 transcript (Fig. 1*A*), indicating that the corresponding 5′ PepT1 sequence is absent from the pgPepT1 transcript.

A 626-bp pgPepT1 cDNA[3] was isolated from the pineal gland by 5′-RACE and found to have a 96-bp 5′-untranslated region followed by a 453-bp open reading frame. The 5′ end (nt 1–105) corresponds to sequence in the 3′ region of intron 20 of the *PepT1* gene (Fig. 1*C*); nt 97–105 of this sequence encodes the first three residues (MVQ) in the deduced protein (Fig. 1*B*). The downstream sequence (nt 106–549) of pgPepT1 transcript is identical to nt 1741–2184 of the intestinal PepT1 cDNA (Fig. 1*B*), which encodes the C-terminal three TMDs region. Accordingly, the pgPepT1 transcript is predicted to encode a 150-amino acid protein (16.7 kDa) corresponding to the C-terminal three TMD of PepT1 protein and three N-terminal residues not found in PepT1 (Fig. 1, *B* and *C*).

To test this prediction, pgPepT1 cDNA was expressed in CHO cells; this generated a protein of ~16 kDa that was detected by an antiserum directed against a C-terminal PepT1 peptide sequence (Fig. 1*D*). These data suggest that pgPepT1 mRNA is a product of the *PepT1* gene and is generated by either alternative splicing or activation of a promoter in intron 20.

pgPepT1 Tissue and Cellular Distribution—pgPepT1 mRNA is not detected by Northern blot analysis in pituitary, retina, or eight brain regions collected at midday and midnight (Fig. 2*A*) nor in eight peripheral tissues collected at midnight (small intestine, kidney, spleen, ovaries, testis, heart, and lung; data not shown).

The indication that expression of pgPepT1 is limited to the pineal gland at night was extended by *in situ* hybridization analysis of brain sections (Fig. 2*B*). This confirmed the pineal and night restricted pattern of expression. In addition, examination of the *in situ* images revealed that the hybridization signal varied in intensity within each section, exhibiting a dense reticular pattern surrounding less dense follicular areas; furthermore, the signal was located entirely above pinealocytes and was not associated with either interstitial cells or endothelial cells in the perivascular space (Fig. 2*C*).

pgPepT1 protein expression was detected in crude rat pineal membrane fractions collected at midnight, as a 16-kDa immunopositive band (Fig. 2*D*), in agreement with the predicted molecular mass calculated from pgPepT1 mRNA sequence and with the size of the immunopositive protein identified in CHO cells transfected with pgPepT1 (Fig. 1*D*). A similar band was not present in crude membrane fractions prepared from cerebellum (Fig. 2*D*) and five other tissues collected at midnight (retina, cortex, pituitary, hindbrain, and midbrain; data not shown); this is in agreement with the restricted expression pattern of pgPepT1 mRNA (Fig. 2*A*). Moreover, this band is selectively enriched in the 100,000 × *g* membrane-containing fraction (Fig. 2*D*), consistent with the three predicted transmembrane domains of the expressed protein. This supports the conclusion that pgPepT1 protein is membrane-associated. Two additional immunopositive bands (~40 and ~60 kDa) were detected in membrane fractions prepared from pineal gland as well as from other brain areas (data not shown) and appear to be nonspecific because pgPepT1 mRNA is not expressed in extrapineal sites.

Daily Rhythm in pgPepT1 mRNA and Protein—pgPepT1 mRNA levels are detectable 5 h after lights off (midnight,

[3] The nucleotide sequence of pgPepT1 cDNA has been submitted to GenBank™ (accession number AY860424).

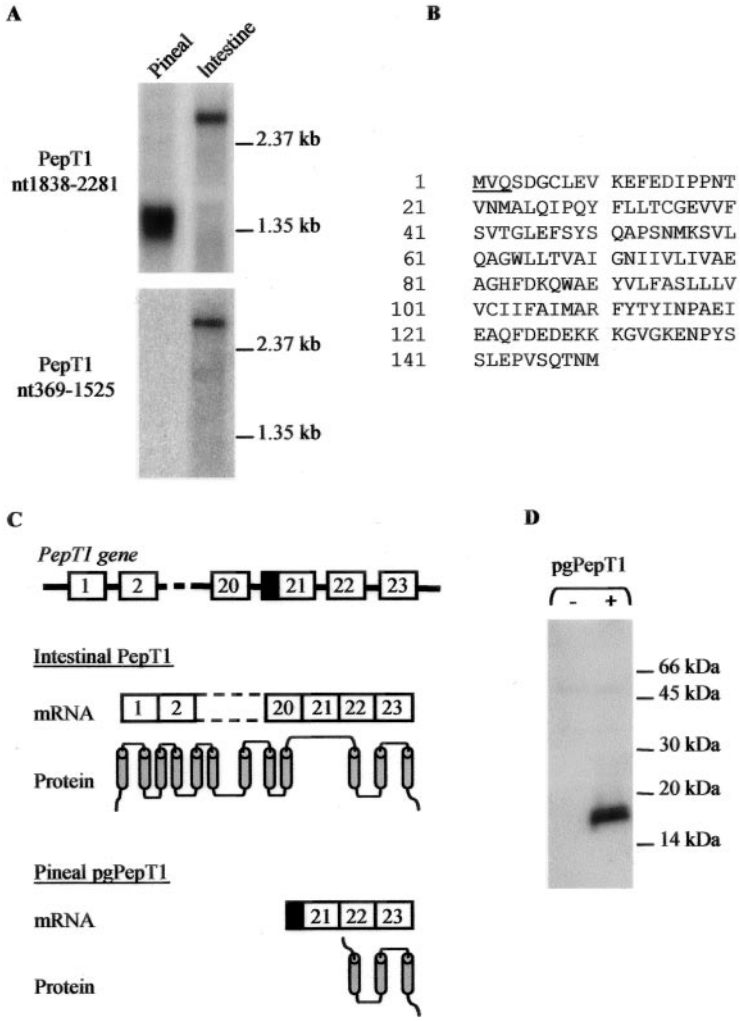

FIG. 1. **Molecular characterization of a novel *PepT1* gene product, pgPepT1, expressed in rat pineal gland at night.** *A*, expression of a novel *PepT1* gene product in pineal gland at night. Northern blot analysis performed on total RNA extracted from pineal glands collected at midnight (ZT19) and from small intestine. Rats were housed in controlled lighting (LD 14:10). RNA preparation and Northern blot analysis were performed as described under "Experimental Procedures." The blot was hybridized with a probe covering either the 3′ (nt 1838–2281) (*top panel*) or the 5′ (nt 369–1525) (*lower panel*) region of the intestinal PepT1 mRNA. Each *lane* contains 10 μg of total RNA. *B*, deduced amino acid sequence of rat pgPepT1. pgPepT1 cDNA,[3] isolated by 5′-RACE, encodes a 150-amino acids protein. The three first N-terminal residues are unique (*underlined*), but the 4–150 amino acids are identical to the 564–710 residues of rat intestinal PepT1 protein. *C*, schematic representation of the intestinal and pineal *PepT1* gene products. The intestinal PepT1 mRNA encloses the 23 exons (*white boxes*) of *PepT1* gene and encodes a 710-amino acids protein with 12 TMDs. The pineal pgPepT1 mRNA corresponds to the last three exons (21–23) of *PepT1* gene. Its 5′ end is part of the intronic sequence (*black box*) immediately upstream the exon 21. pgPepT1 encodes an N-truncated form of the intestinal PepT1 protein, with three predicted TMDs. *D*, Western blot analysis of pgPepT1 protein expressed in heterologous system. Protein lysates from CHO cells untransfected (−) or transfected (+) with pgPepT1 cDNA expression vector were separated by SDS-PAGE, transferred to nitrocellulose, and immunodetected with a rabbit polyclonal antibody against the C-terminal 15 amino acids of rat PepT1.

ZT19) and reach a maximum 1 h before lights on (ZT23) (Fig. 3*A*); they rapidly decrease thereafter and are undetectable at midday and late afternoon. The amplitude of this daily rhythm is >100-fold. pgPepT1 protein levels change in parallel, with high values at ZT19, ZT23, and ZT3; however, pgPepT1 protein does not disappear during the day; accordingly, the amplitude of the rhythm is ~ 9-fold (Fig. 3*A*). In comparison, the synaptophysin immunosignal was constant through the LD cycle (Fig. 3*A*).

pgPepT1 mRNA Abundance Is under Circadian and Photoneural Control—A 24-h rhythm in pgPepT1 mRNA expression persisted in animals that had been housed in constant darkness for 3 days (DD) (Fig. 3*B*), indicating that the daily rhythm in pgPepT1 mRNA expression does not require dark/light transitions, but is circadian in nature. The rhythm was absent in animals maintained in constant light (LL) (Fig. 3*B*), which blocks SCN stimulation of the pineal gland. The nocturnal induction of pgPepT1 transcript was also not detected in pineal glands removed from animals in which the SCN → pineal circuit was disrupted by surgical removal of superior cervical ganglia (*SCGX*) or decentralization (*DCNT*) (Fig. 4*A*). These observations are consistent with the interpretation that the nocturnal increase in pgPepT1 mRNA in the pineal gland is driven by the well described SCN → pineal pathway (2).

pgPepT1 mRNA Abundance Is Elevated by an Adrenergic Mechanism—As indicated in the introduction, NE is released at night from the pineal sympathetic afferents in response to SCN stimulation. Recent microarray studies indicate that 1-h NE treatment in pineal organ culture increases *PepT1* gene expression (17). Here, it was found that injection of NE to SCGX animals induced pgPepT1 expression (Fig. 4*A*). SCGX animals were used because this procedure removes sympathetic terminals in the pineal gland, thereby allowing circulating NE to gain access to pinealocytes (29).

Intact animals were also treated with the β-adrenergic receptor-selective agonist ISO, which is not taken up into sympathetic nerve endings. This treatment increased pgPepT1 expression in the pineal gland but not in other areas of the brain, as detected by *in situ* hybridization (Fig. 4*B*).

NE treatment also increased the abundance of pgPepT1 mRNA in pineal organ culture in a time-dependent manner (Fig. 5*A*). An increase was detected by Northern blot analysis within 2 h of NE stimulation and peaked at >100-fold control levels at 6 h. The NE-induced increase in pgPepT1 protein generally paralleled changes in pgPepT1 mRNA (Fig. 5*A*); the maximal response (~5-fold) occurred 6 h after NE treatment (Fig. 5*A*). These findings indicate that NE elevates *PepT1* gene expression through a direct action on the gland.

As observed in *in vivo* experiments (Fig. 3*B*), the size of pgPepT1 transcripts decreased during NE treatment (Fig. 5*A*). We found this size difference disappeared when the transcripts were experimentally deadenylated using an oligo(dT)/RNase H assay (Supplementary Material, Fig. S1). This suggests that deadenylation of the pgPepT1 transcript occurs during the course of the NE stimulation as previously observed for the rat AANAT transcript (6).

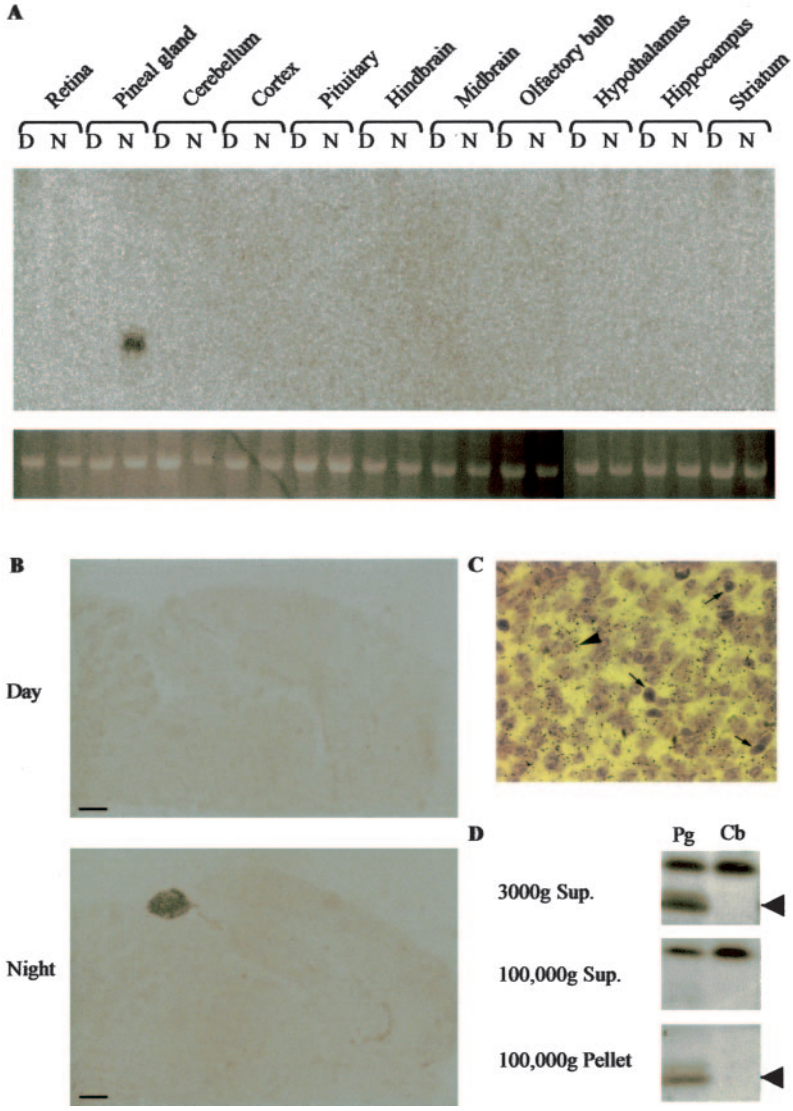

FIG. 2. **Tissue-specific expression of pgPepT1.** *A*, Northern blot analysis performed on total RNA obtained from tissues collected at midday (ZT7, *D*) and midnight (ZT19, *N*). Rats were housed in controlled lighting (LD 14:10). RNA preparation and Northern blot analysis were performed as described under "Experimental Procedures." The blot was hybridized with a probe covering the region between nt 1838 and 2281 of the intestinal PepT1 mRNA. Each *lane* contains 15 μg of total RNA. As loading control, the ethidium bromide-stained 18 S UV picture is presented. *B*, *in situ* hybridization on rat brain median sections with a ^{35}S-labeled antisense 38-mer oligoprobe detecting pgPepT1 mRNA. The top section (*Day*) is from an animal killed at ZT6, and the bottom section (*Night*) is from an animal killed at ZT18. Rats were housed in controlled lighting (LD 12:12). *Bar* = 1 mm. *C*, microautoradiograph showing part of a rat pineal gland. The section was hybridized with a pgPepT1 ^{35}S-labeled oligonucleotide probe and covered by liquid emulsion. The grains are observed above the pinealocytes (*arrowhead*) and not above the interstitial cells (*arrow*) recognized by their darker stained nuclei. *D*, immunodetection of pgPepT1 protein in pineal crude membrane fraction. Tissue homogenates were prepared from pineal gland (*Pg*) and cerebellum (*Cb*) collected at midnight. The protein lysates were first centrifuged at 3,000 $\times$ *g*, and the supernatants were further centrifuged at 100,000 $\times$ *g*. The 3,000 $\times$ *g* and 100,000 $\times$ *g* supernatants (*Sup.*), as well as the 100,000 $\times$ *g* pellets, were analyzed by Western blot using a rabbit polyclonal antibody against the C-terminal 15 amino acids of rat PepT1. A 16-kDa immunopositive band (*arrowhead*) was specifically detected in the 100,000 $\times$ *g* pineal membrane-containing fraction.

ISO treatment of cultured pineal glands also increased pgPepT1 transcript levels (Fig. 5*B*), as did treatment with *R*-(−)-phenylephrine, the α_1-adrenergic receptor-selective agonist, albeit to a lesser degree (Fig. 5*B*). These results suggest that the effects of NE on pgPepT1 mRNA are mediated by β-adrenergic receptors and that α_1-adrenergic receptors appear to play a lesser role.

NE stimulation of pinealocytes is known to elevate cAMP content through a β-adrenergic/α_1-adrenergic "AND" gate mechanism (2). Here, we demonstrated that two cAMP protagonists, dibutyryl cyclic adenosine monophosphate (Bt_2cAMP) and forskolin, mimic the effect of NE on pgPepT1 mRNA (Fig. 5*B*). In addition, the protein kinase A antagonists, (R_p)-8-PIP-cyclic AMP, H89, and KT5720, inhibited the NE-stimulated pgPepT1 mRNA expression (Fig. 5*C*). These data indicate that cAMP mediates the stimulatory effects of NE on pgPepT1 transcription through a protein kinase A-dependent mechanism.

Functional Characterization of the Putative Intronic pgPepT1 Promoter—As indicated above, pgPepT1 could result either from alternative splicing or from the utilization of an alternative internal promoter. Sequence analysis and comparison of pgPepT1 cDNA with the *PepT1* gene suggest that a potential alternative promoter is present in the intron 20, because part of the 5′-untranslated region of pgPepT1 transcript corresponds to this intronic sequence. Manual and computer (MatInspector (30)) analysis of intron 20 (546 bp) identified a potential TATA box near the pgPepT1 transcription start site, and several other cis-elements, including at least two putative cAMP-responsive element (CRE) related sequences, an inverted CCAAT box (CCAATinv) and a cluster of three pentamers matching the consensus sequence TAAT(C/T) (Fig. 6*A*). The latter are of special interest, because these are closely related to the photoreceptor conserved element (PCE) (31, 32) and identical to the previously described pineal gland-regulatory element (33). These sequences bind the cone rod homeobox (Crx) transcription factor (33–35), which is linked to photoreceptor and pineal-specific gene expression (34–37). Here, PCE is used to identify TAAT(C/T) sequences (Fig. 6*A*).

To determine whether the intron 20 of *PepT1* gene contains a proximal promoter for pgPepT1, the promoter activity of intron 20-luciferase reporter construct was monitored in pinealocytes and COS-7 cells (Fig. 6, *B* and *C*). Intron 20 was found to confer a strong induction of reporter activity in response to cAMP (Fig. 6*C*). Bt_2cAMP treatment increased expression by about 8- and 6-fold in pinealocytes and COS-7 cells, respectively (Fig. 6*C*). This data suggest that the intron 20 of *PepT1* gene functions as an alternative promoter, which is responsive to cAMP. Interestingly, the response to Bt_2cAMP was 50% lower in pinealocytes transfected with the construct "Intron20-PCE," lacking the three PCEs in the distal 128 bp of the 5′ promoter sequence (Fig. 6, *B* and *C*), whereas there was no significant difference in expression in COS-7 cells (Fig. 6*C*).

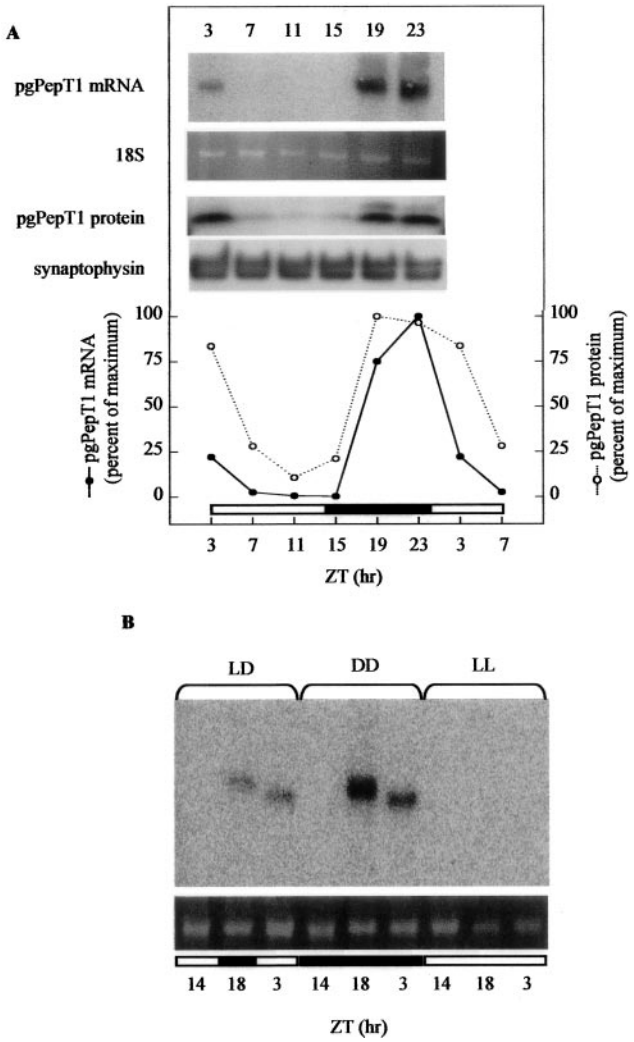

FIG. 3. **Rat pineal pgPepT1 exhibits a 24-h rhythm *in vivo* that is under circadian-photoneural regulation.** *A*, daily rhythm of pgPepT1 mRNA and protein in rat pineal gland. Animals were housed in controlled lighting (LD 14:10) and killed at 4-h intervals. Pineal glands were collected, and total RNA and crude membrane fractions were prepared as described under "Experimental Procedures." *Top panel* presents the Northern blot hybridized with a probe covering part of the intestinal PepT1 mRNA (nt 1838–2281). Each *lane* contains 5 μg of total RNA. As loading control, a 28 S UV picture is presented. *Lower panel* shows the Western blot immunodetected with the anti-rat PepT1 serum. Each *lane* corresponds to the crude membrane protein fractions prepared from a pool of four pineal glands. The immunoblotting with anti-synaptophysin serum is also shown as a loading control. The *graph* plots the abundance of pgPepT1 transcript (*filled circles*) and protein (*open circles*) levels, expressed as percentage of the maximum. The ZT3 and ZT7 values have been plotted twice. *Open bars* and the *closed bar* show light and dark phases, respectively. *B*, pgPepT1 mRNA rhythm is circadian in nature. Shown is the Northern blot analysis of pgPepT1 expression in pineal glands collected at ZT14, ZT18, and ZT3 from rats under normal lighting (*LD*), constant darkness (*DD*), and constant light (*LL*) conditions. Animals were housed in controlled lighting (LD 14:10) or maintained for 3 days in DD or LL and killed at ZT14, ZT18, and ZT3. RNA preparation and Northern blot analysis were performed as described under "Experimental Procedures." Each *lane* contains 5 μg of total RNA. As loading control, an 18 S UV picture is presented. *Open bars* and *closed bars* show light and dark phases, respectively.

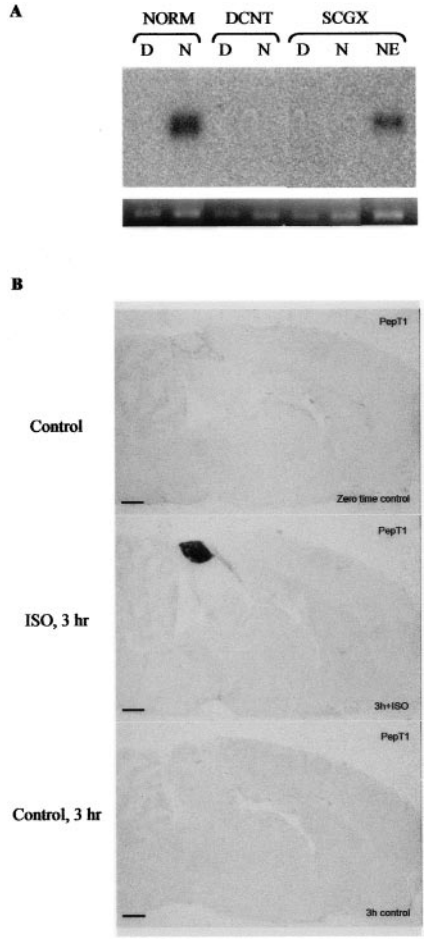

FIG. 4. **Regulation of pgPepT1 expression *in vivo*.** *A*, Blockage of the central stimulation of the pineal gland abolishes the nocturnal induction of pgPepT1 mRNA expression. Pineal RNA were obtained from normal (*NORM*), decentralized (*DCNT*), and superior cervical ganglionectomized (*SCGX*) rats, killed at midday (ZT7, *D*) and midnight (ZT19, *N*). In addition, one group of SCGX rats was injected with 1 mg/kg NE at ZT16 and killed 3 h later at midnight (ZT19, *NE*). Rats were housed in controlled lighting (LD 14:10). Each *lane* contains 3 μg of total RNA. As loading control, an 18 S UV picture is presented. *B*, isoproterenol injection induces pgPepT1 mRNA expression in the pineal gland. Shown is the *in situ* hybridization using pgPepT1 ^{35}S-labeled oligonucleotide probe on median sections of brains collected from a rat killed at ZT6 (*Control*), from a rat injected intraperitoneally with 5 mg of isoproterenol at ZT6, ZT6.5, and ZT7.5 and killed at ZT9 (*Iso*, 3 h), and from a rat injected intraperitoneally with physiological saline in the same conditions and killed at ZT9 (*Control*, 3 h). Rats were housed in controlled lighting (LD 12:12). *Bars* = 1 mm.

pgPepT1 Has Transporter Modulator Activity—The effect of pgPepT1 on Gly-Sar transport was studied using human retinal pigment epithelial cells, which do not constitutively express peptide transport activity. Transfection of the pgPepT1 expression vector alone did not change Gly-Sar transport activity compared with the control (Fig. 7), indicating that pgPepT1 does not confer peptide transport activity. In the same system, transfection of human PepT1 expression vector confers Gly-Sar transport activity (267.4 pmol/10^6 cells/15 min; Fig. 7). Co-transfection of pgPepT1 with PepT1 reduced transport activity by ~85% (Fig. 7). This does not appear to reflect nonspecific toxic effects of pgPepT1 transfection, because it did not decrease transport conferred by transfection with mouse PepT2 (Fig. 7). Accordingly, although pgPepT1 by itself has no peptide transport activity, it modulates the transporter activity of PepT1.

DISCUSSION

The discovery of a new gene product that is selectively expressed in a tissue under tight regulation has fundamental biological interest. This is the case in this report, in which we have found a novel product of the *PepT1* gene that is exclusively expressed in the pineal gland. It is of added interest that there is a daily rhythm in expression. Three aspects of this advance will be discussed below: (i) the products of *PepT1* gene

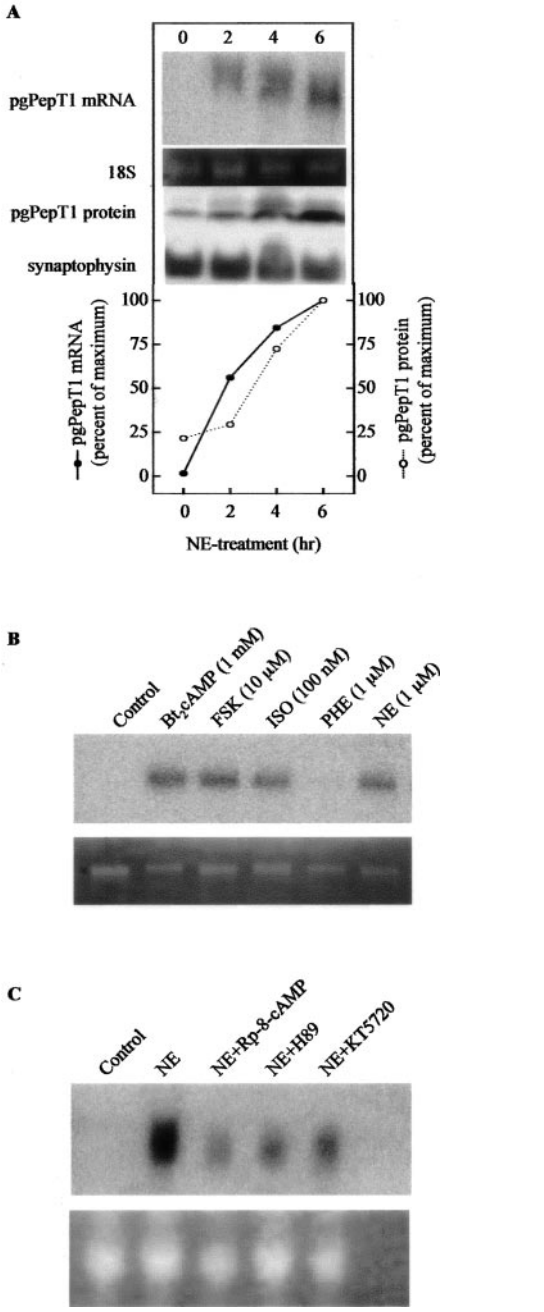

FIG. 5. **Regulation of pgPepT1 expression *in vitro*.** *A*, NE increases pgPepT1 mRNA and protein levels in time-dependent manner *in vitro*. *Top panel* presents the Northern blot analysis of pgPepT1 mRNA expression in cultured pineal glands exposed to norepinephrine (*NE*, 1 μM) for the indicated times. RNA preparation and Northern blot analysis were performed as described under "Experimental Procedures." Each *lane* contains 5 μg of total RNA. As loading control, an 18 S UV picture is presented. *Lower panel* shows the Western blot immunodetected with the anti-rat PepT1 serum. Each *lane* corresponds to the crude membrane protein fractions prepared from a pool of four cultured pineal glands exposed to NE (1 μM) for the indicated times. The immunoblotting with anti-synaptophysin serum is also shown as a loading control. The *graph* plots the quantification of pgPepT1 transcript (*filled circles*) and protein (*open circles*) levels, expressed as percentage of maximum. *B*, effect of adrenergic and cyclic AMP agonists on pgPepT1 mRNA expression. Primary rat pineal cells were incubated with different drugs (Bt_2cAMP, dibutyryl cAMP; *FSK*, forskolin; *ISO*, isoproterenol; *PHE*, phenylephrine; *NE*, norepinephrine) at the indicated concentration for 6 h. Each *lane* contained 5 μg of total RNA. As loading control, an 18 S UV picture is presented. *C*, effect of protein kinase A antagonists on NE-stimulated pgPepT1 mRNA expression. Pineal glands were cultured for 1 h in presence of (R_p)-8-PIP-cyclic AMP (500 μM) or H-89 (10 μM) or KT5720 (3 μM); NE (1 μM) was then added and incubation was continued for 6 h in presence of the indicated drugs.

and their specific tissue and temporal expression, (ii) the regulation of *PepT1* gene expression by alternative promoters, and (iii) the potential function of pgPepT1 protein.

PepT1 Gene Products and Their Tissue and Temporal Expression—The findings of this report and published studies indicate that *PepT1* gene is the source of at least three transcripts, each with a highly specific tissue distribution. The first described PepT1 transcript encodes a protein of ~700 amino acids with 12 predicted TMDs; it is predominantly expressed at the brush-border membrane of epithelial cells in the small intestine and, to a lesser extent, in the kidney proximal tubule S1 segment (25, 26, 38). A closely related transcript, PepT1-regulating factor (PepT1-RF), has been isolated from a human duodenum cDNA library (39); it encodes a 200-amino acid C-truncated PepT1 isoform, encompassing the first 5 TMDs. Here, we identified a novel *PepT1* gene product, selectively expressed in the pineal gland, encoding a 150-amino acid protein identical to the C-terminal 3-TMD region of the intestinal PepT1, with the addition of 3 N-terminal residues.

A common feature of both intestinal PepT1 and pineal pgPepT1 transcripts is a daily pattern in expression. However, there are notable differences. One is the magnitude of the daily rhythm, ~4-fold for the intestinal PepT1 transcript (40), and ~100-fold for the pineal pgPepT1 mRNA. A second is that the intestinal PepT1 mRNA levels peak at the end of the day (40), whereas the peak in pgPepT1 expression occurs at night. In addition, different regulatory mechanisms control these rhythms. Food intake is an important factor for setting the diurnal rhythm of intestinal PepT1 (41, 42), whereas the endogenous SCN clock drives the night-day oscillation in pgPepT1 expression, through the nocturnal activation of the adrenergic-cAMP signaling pathway. Accordingly, it would appear that, whereas both rhythms are influenced by environmental factors, the mechanisms underlying these rhythms are distinctly different, as are the ultimate gene products, suggesting to us that unrelated factors selected for the evolution of these gene products and their regulatory mechanisms.

Regulation of PepT1 Gene Expression by Alternative Promoters—The generation from a single gene of such protein diversity in a tissue- and temporal-specific manner implies a tightly regulated transcriptional system. Here, we have found that the mechanism underlying the regulation of *PepT1* gene expression involves the utilization of an alternative promoter, rather than alternative splicing.

The proximal P1 promoter located upstream of the exon 1 controls PepT1 expression in intestinal cells, leading to generation of the full-length PepT1 protein (43, 44). It has been previously suggested that the P1 PepT1 promoter is regulated by selective amino acids and dipeptides present in the diet (43), although it is still unclear how it mediates the diurnal intestinal PepT1 expression.

pgPepT1 expression occurs in response to the activation of an alternative P2 promoter, located 40 kb downstream of the P1 promoter, in intron 20. This intronic P2 promoter governs rhythmic pgPepT1 expression via its ability to respond to cAMP, thus mediating circadian clock transcriptional control. The molecular mechanism that triggers the cAMP-dependent activation of pgPepT1 P2 promoter may be similar to that of other genes expressed in the rat pineal gland on a 24-h basis. Previous studies have shown that rat pineal-specific 24-h AANAT transcription is regulated by a combination of an inverted CCAAT box and CRE element (45). These transcription factor binding sites are also present in the P2 pgPepT1 promoter, and in future studies it will be necessary to determine their involvement in regulating the 24-h pgPepT1 pattern of expression.

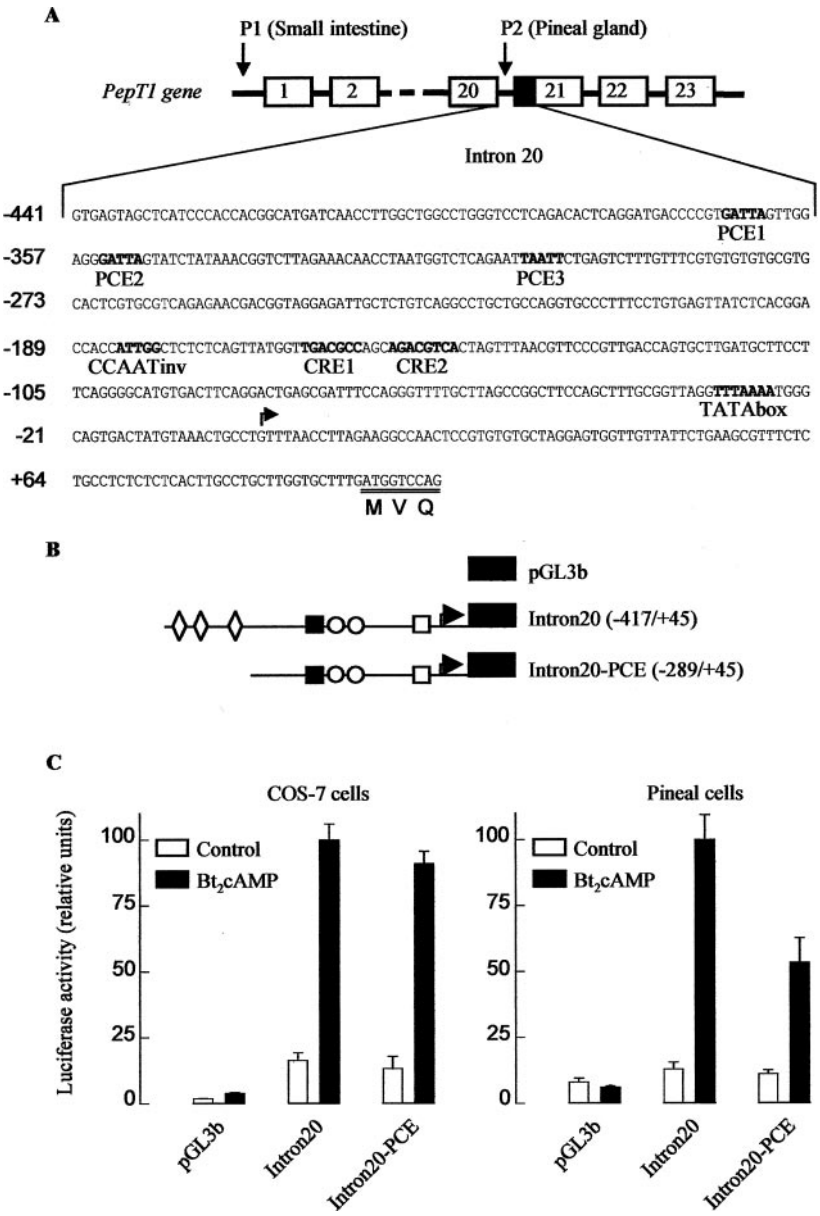

FIG. 6. **Structural and functional analysis of the intronic pgPepT1 promoter.** *A*, description of the intron 20 of rat *PepT1* gene. Shown is the schematic representation of the alternative promoter (P2) located in the intron 20 of *PepT1* gene. Bellow is presented the nucleotide sequence of the intron 20 (GenBank™ accession number NW047456). Putative cis-regulatory elements (*PCE*, photoreceptor conserved element; *CCAATinv*, inverted CCAAT box; *CRE*, cAMP-responsive element), identified manually or with the MatInspector software program, are shown in *bold*. The location of a potential TATA box and the pgPepT1 mRNA[3] transcription start site (*arrow*) are also indicated. The nine first nucleotides of the coding sequence (*underlined*) are located at the 3′ end of the intron 20. *B*, schematic representation of the promoter/reporter constructs. DNA fragments of the intronic promoter P2 (*Intron20*, −417/+45; *Intron20-PCE*, −289/+45) were subcloned into the promoterless pGL3-basic vector (pGL3b), upstream of the firefly luciferase coding sequence (Luc, *black box*). ◇, PCE; ■, CCAATinv; ○, CRE; □, TATA box. *C*, promoter activity of the intron 20 of rat *PepT1* gene. COS-7 cells and primary rat pineal cells were transfected with the indicated promoter-reporter construct. The next day, cells were stimulated with 1 mM Bt_2cAMP or left untreated (control). Cells were harvested 24 h later and assayed for luciferase activity. The *graph* illustrates the results of a representative experiment. Data are expressed as the mean ± S.E. of triplicate determinations. Similar results were obtained in three independent experiments.

It is likely that the remarkable tissue specificity observed with pgPepT1 reflects the presence in the P2 promoter of several PCEs. As indicated above, these binding sites interact with members of the orthodenticle homeobox/Crx family of transcription factors to mediate retinal- and pineal-specific gene expression (34–37, 46), a reflection of the common origin of retinal photoreceptors and pinealocytes from an ancestral photoreceptor cell (47). The Crx target genes include AANAT (32, 33, 37), hydroxyindole-*O*-methyltransferase (35), a pineal night-specific ATPase (PINA) (33), and several phototransduction genes (37). It is notable that deletion of a sequence containing multiple PCEs reduces the transcriptional activity of the pgPepT1 P2 promoter in pineal cells but not in COS-7 cells. This suggests that Crx or a related transcription factor in the pinealocyte enhances cAMP-dependent expression. The finding that the fragment of pgPepT1 promoter used in this study mediated expression in COS-7 cells, however, indicates that this fragment may not contain all the information required to control pineal restricted expression; rather, it is possible that other sequence in the *PepT1* gene functions to suppress expression in non-pineal cells. This type of silencing has previously been observed to influence pineal restricted gene expression of the AANAT-2 gene in zebrafish (32). Further investigations should elucidate the mechanism underlying the tissue-specific repression of pgPepT1 expression.

Function of the Novel pgPepT1 Isoform—It is remarkable that the *PepT1* gene triggers the expression of transcripts that encode a 12-, a 5-, and a 3-TMD protein, each with different functional properties. The intestinal full-length PepT1 protein operates as a proton/peptide symporter responsible for the nutritional uptake of di- and tri-peptides (21); the C-truncated isoform, PepT1-RF, doesn't exhibit transport activity but acts as a pH-sensing regulatory factor that modulates transport activity of PepT1 (39); pgPepT1 also appears to have transporter modulatory activity. Although the target of pgPepT1 in the pineal gland is not clear at this time, the temporal pattern of expression suggests to us that it plays a role in circadian pineal physiology, perhaps enhancing a function that is important for the increase in melatonin production at night. Initial efforts toward the identification of a target have failed to reveal an effect on $[Ca^{2+}]_i$ and on tryptophan or histidine uptake (data not shown).

Another possibility that should be considered is that pgPepT1 forms a functional PepT1-like transporter, through oli-

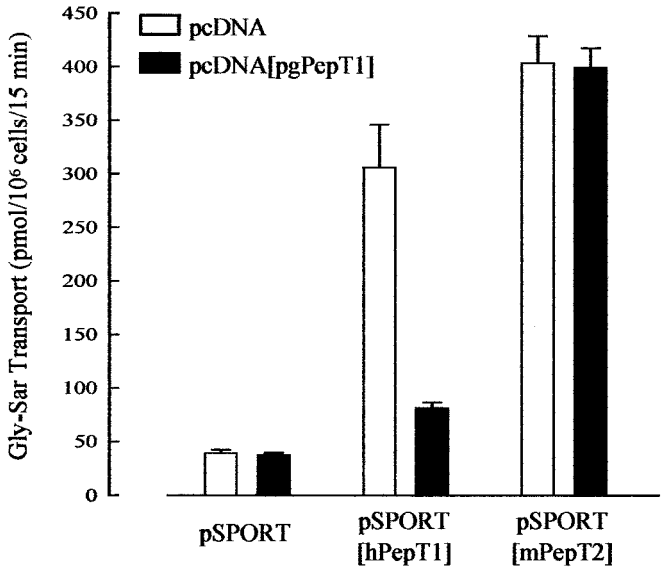

FIG. 7. **Coexpression of pgPepT1 decreases PepT1 transport activity.** pgPepT1 was expressed in human retinal pigment epithelial cells alone or in combination with hPepT1 or mPepT2 by the vaccinia virus expression system. The amount of plasmid DNA was kept constant at 2 μg/assay with empty vector. Transport activity was measured for 15 min at 37 °C using 50 μM [^{14}C]Gly-Sar as the substrate. Transport buffer consisted of 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 5 mM glucose, buffered with 25 mM 4-morpholineethanesulfonic acid/Tris, pH 6.0. Data (means ± S.E.) are from two independent transfections, each done in triplicate.

gomerization; this is seen with other membrane proteins, especially ion channels and pumps (48). A peptide transporter in the pineal gland could contribute to clearance of active or degraded neuropeptides, peptides in the circulation, or peptides generated by extracellular proteases. However, under the experimental conditions described here, pgPepT1 protein is devoid of dipeptide transport activity; similar results were obtained in CHO cells (data not shown).

The prediction that pgPepT1 might regulate the function of another membrane protein is supported by the finding that pgPepT1 modulates PepT1 transport activity. Although PepT1 is not present in pinealocytes, it is reasonable to suspect that pgPepT1 can bind to and regulate the function of another membrane protein expressed in the pineal gland. Indeed, the daily rhythm in pgPepT1 could confer circadian control on a stably expressed transporter. An example of this type of regulatory mechanism comes from *D. melanogaster* in which the protein SLOB is produced on a circadian basis and influences the slowpoke calcium-dependent potassium channel (dS10) through direct protein-protein interactions (49, 50).

The main function of the pineal gland is to generate a rhythm in melatonin. This involves the global activation of pineal metabolism, *i.e.* transport, uptake, membrane physiology, ion channel activation, Na^+/K^+-ATPase pump activity, and signaling. In addition to recognized transcriptional regulation, some of these processes may also be influenced through pgPepT1 binding.

Alternative Promoter Regulation of Gene Expression in the Pineal Gland—Alternative promoters allows innovation, diversity, and flexibility in the regulation of gene expression, especially as in controlling tissue specificity (51). The advances described in this report add to this body of evidence, in indicating that distinct protein isoforms can be expressed from a single gene in unrelated tissues, under the control of entirely different mechanisms.

It is of interest to note that the studies of the pineal gland have yielded other examples of the use of alternative promoters. These include the inducible cAMP early repressor, product of the cAMP-responsive element modulator gene (8, 52), PINA (pineal night-specific ATPase), generated from *ATP7B* gene disrupted in Wilson disease (11, 33) and HIOMT (22). In the case of the first two, this led to the circadian production of protein isoforms lacking a large N-terminal portion, exhibiting different functions from the full-length proteins. In the future, it is predictable that the development of novel strategies, for example exon junction microarrays (53), will provide additional examples of novel mRNA variants produced by alternative mRNA splicing and promoter usage, and how they control protein diversity and tissue-specificity.

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